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(54) Title: <b>NOVEL ALKALINE CELLULASES</b>  (57) Abstract  Cellulases selected from the group consisting of Family 7 cellulases and variants of these cellulases comprising a core and optionally a C-terminal link consisting of 10 amino acids at the most, especially cellulases having tryptophan, tyrosine or phenylalanine in position 55 relative to the sequence alignment of Figure 5 and/or cellulases having a substrate binding cleft of a depth of at least 12 Å, exhibit enhanced enzyme activity in the alkaline pH range while exerting a moderate cellulolytic action on the cellulosic substrate and are, for example, useful in detergent compositions, especially for soil removal or colour clarification or preventing backstaining; in fabric softeners; for bio-polishing of textiles; for drainage improvement of paper pulp; for plant degradation. Cellulases from <i>Humicola insolens</i> , <i>Fusarium oxysporum</i> , <i>Trichoderma reesei</i> and <i>Myceliophthora thermophila</i> are referred to.		

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## 5 NOVEL ALKALINE CELLULASES

The present invention relates to a cellulase capable of removing soil from fabric, a detergent composition comprising the cellulase, a method of treating soiled fabric with the cellulolytic enzyme, and use of the cellulase e.g. in detergent compositions, in fabric softeners, for colour clarification of textile fabrics (removal of fluffs and pills), for preventing backstaining in washing of fabric, for soil removal, for deinking of used paper, and for pulp recycling.

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## BACKGROUND OF THE INVENTION

Repeated washing of fabrics, especially cellulose containing fabrics, generally causes a harshness in the fabric used.

The use of cellulases, i.e. cellulolytic enzymes, for harshness reduction of cellulose containing fabrics, e.g. cotton, was suggested and demonstrated a long time ago.

The practical exploitation of cellulases has, to some extent, been set back by the nature of the known cellulase preparations which are often complex mixtures of a variety of single cellulase components, and which may have a rather low specific activity. It is difficult to optimise the production of single components in multiple enzyme systems and thus to implement industrial cost-effective production of cellulases, and their actual use has been hampered by difficulties arising from the need to employ rather large quantities of the enzymes to achieve the desired effect.

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The drawbacks of previously suggested cellulases may be remedied by using single-component enzymes selected for a high specific activity. Single-component cellulases are described in, e.g. WO 91/17243, WO 91/17244 and WO 91/10732.

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E.g. in WO 91/17244 is disclosed a cellulose-degrading enzyme (a cellulase) derivable from a fungus other than *Trichoderma* or *Phanerochaete* which comprises a carbohydrate binding domain homologous to a terminal A region of *Trichoderma reesei* cellulases, the carbohydrate binding domain being capable of effecting binding of the enzyme to an insoluble cellulosic substrate, which may be employed for textile treatment, e.g. for reducing the harshness of cotton-containing fabrics and for soil removal and colour clarification of fabrics. In example 3 and fig. 13 is disclosed the preparation of a *Fusarium oxysporum* C-family endoglucanase and the DNA sequence and derived amino acid sequence thereof, respectively. Later it was found that the disclosed amino acid sequence was not correct; the corrected sequence is published in Sheppard, P.O., Grant, F.J., Oort, P.J., Sprecher, C.A., Foster, D.C., Hagen, F.S., Upshall, A., Mcknight, G.L. and Ohara, P.J.: The use of conserved cellulase family-specific sequences to clone cellulase homolog cdnas from fusarium-oxysporum. *Gene*. 150:163-167, 1994. In example 4 and Fig. 14A-E is disclosed the preparation of a *Humicola insolens* endoglucanase 1 (EG I) and the DNA sequence and derived amino acid sequence thereof, respectively. Further, in example 4 (page 32, line 1 to 5) is described the construction of expression plasmid of a truncated EG I (denoted EG I') wherein the last 13 amino acids of the coding region were eliminated and the altering of Val to Leu in position 421 (position 401 in the sequence of the enzyme). The gist of the invention disclosed in WO 91/17244 is to provide a cellulase which, besides the enzyme core, has a carbohydrate binding domain (CBD) which is homologous to the A region of *Trichoderma reesei* cellulases, since the function of the CBD in the enzyme molecule was believed to be to mediate binding to solid substrates including cellulose and consequently to enhance the activity of such enzymes towards such substrates.

The problem underlying the present invention is to obtain single-component endoglucanases having enhanced enzyme activity in the alkaline pH range, while at the same time exerting a moderate cellulolytic action on the cellulosic substrate. In other words, the endoglucanase should neither destroy the cellulosic substrate as such. For example, when the substrate is a cellulosic fabric, the used endoglucanase should not result in a substantial tensile strength loss of the fabric. The enhanced alkaline activity of the enzyme is also essential, since most applications of endoglucanases advantageously take place in the alkaline pH range.

#### SUMMARY OF THE INVENTION

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Surprisingly, it has now been found that certain cellulases which do not comprise a carbohydrate binding domain, i.e. essentially consist of the core enzyme, or which at least do not comprise a carbohydrate binding domain which is homologous to the A region of *Trichoderma reesei* cellulases, may have an enhanced activity. This may for example result in improved soil removal from fabrics.

Preferably, the cellulases of the invention are endoglucanases which have the amino acid residue tryptophan (Trp or W) in the position corresponding to position 55 of the structural homology frame in Figure 5.

More specifically, it has been found that cellulases selected from the group consisting of cellulases classified in family 7 as described in Henrissat, B. et al.: Biochem. J. (1993), 293, p. 781-788, and cellulase variants derived from a parent cellulase classified in family C, comprising a core and optionally a C-terminal link consisting of 10 amino acids at the most may perform excellent in detergents with respect to soil removal in comparison with the known cellulases.

In one aspect, the invention relates to endoglucanases which may be truncated, e.g. genetically truncated, variants of known endoglucanases, and the use thereof e.g. for washing, cleaning, deinking and pulping purposes.

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In another aspect, the invention relates to cellulases having high activity on cellotriase in the presence of a detergent matrix, high dispersing action on carbon black, and high alkaline activity on acid swollen cellulose at pH 8.

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The present endoglucanases are useful e.g. for soil removal and may thus be applied to detergent compositions, detergent additives and/or fabric softeners.

15 Other uses of the present endoglucanases are for colour clarification of textile fabrics (removal of fluffs and pills), for preventing backstaining in washing of fabric, for soil removal, for deinking of used paper, and for pulp recycling.

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#### DETAILED DESCRIPTION OF THE INVENTION

In the present specification and claims, the term "cellulase" denotes an enzyme that hydrolyses cellulose. The cellulase may be a component occurring in a cellulase system produced by a given microorganism, such a cellulase system mostly comprising several different cellulase enzyme components including those usually identified as e.g. cellobiohydrolases, exo-cellobiohydrolases, endoglucanases,  $\beta$ -glucosidases. Alternatively, the cellulase may be a single component, i.e. a component essentially free of other cellulase components usually occurring in a cellulase system produced by a given microorganism, the single component being a recombinant component, i.e. produced by cloning of a DNA sequence encoding the single component and subsequent cell transformed with the DNA sequence and expressed in a host.

The host is preferably a heterologous host, but the host may under certain conditions also be the homologous host.

In a preferred embodiment of the invention, the cellulase is an endoglucanase.

The term "soil removal" or "particulate soil removal", as used herein, refers to enhanced cleaning of cellulose-containing fabrics or garment, e.g. cotton, contaminated by particles of soil or of other insoluble matter entrapped by microfibrills spreading out on the fibre surface.

In the present context, the term "homologous" or "homologous sequence" is intended to indicate an amino acid sequence differing from those of Figure 1, 2, 3, 4, 5 and 6, respectively, by one or more amino acid residues. The homologous sequence may be one resulting from modification of an amino acid sequence shown in these figures, e.g. involving substitution of one or more amino acid residues at one or more different sites in the amino acid sequence, deletion of one or more amino acid residues at either or both ends of the enzyme or at one or more sites in the amino acid sequence, or insertion of one or more amino acid residues at one or more sites in the amino acid sequence. The modification of the amino acid sequence may suitably be performed by modifying the DNA sequence encoding the enzyme, e.g. by site-directed or by random mutagenesis or a combination of these techniques in accordance with well-known procedures. Alternatively, the homologous sequence may be one of an enzyme derived from another origin than the cellulases corresponding to the amino acid sequences shown in Figure 1, 2, 3, 4, 5, and 6, respectively. Thus, "homologue" may e.g. indicate a polypeptide encoded by DNA which hybridizes to the same probe as the DNA coding for the cellulase with the amino acid sequence in question under certain specified conditions (such as presoaking in 5xSSC and prehybridising for 1 h at -40°C in a solution of 20% formamide, 5xDenhardt's solu-

tion, 50 mM sodium phosphate, pH 6.8, and 50  $\mu$ g of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100  $\mu$ M ATP for 18 h at -40°C). The homologous sequence will normally exhibit a degree of homology (in terms of identity) of at least 50%, such as at least 60%, 65%, 70%, 75%, 80%, 85%, 90% or even 95% with the amino acid sequences shown in Figure 1, 2, 3, 4, 5 and 6, respectively.

10 Preferably, the degree of homology is based on three-dimensional structural homology of the cellulases. For example, the Applicant has prepared the sequence alignment shown in Figure 5, wherein the amino acid sequences of three endoglucanases and one cellobiohydrolase were aligned:

15

EG1-F: *Fusarium oxysporum* endoglucanase EG1  
(fus\_eg1\_nat.pdb, a-chain)

EG1-H: *Humicola insolens* endoglucanase EG1  
(legi.pdb, a-chain)

20 EG1-T: *Trichoderma reesei* endoglucanase EG1  
(no pdb structure)

CBH1: *Trichoderma reesei* cellobiohydrolase  
(1cel.pdb, a-chain)

25 The references in the brackets refer to the Brookhaven Database identification for entries.

Initially the sequences were aligned based on secondary structure homology, but superimposition and visual examination of the X-ray structures of EG1-F, EG1-H and CBH1 necessitated several modifications of the sequence alignment. The final output in Fig. 5 is based on three-dimensional structural alignment.

35 The C-terminals are visible to different extents in the three crystal structures. Following the common sequence motif IGST (Res#445-449), three residues are visible in EG1-



F, one in EG1-H, and five in CBH1. For the sequence comparison in Table 1 below, residues from the N-terminals to the C-terminal motif NPSG in CBH1 are included (Res#453 in Fig. 5), i.e. 402 residues from EG1-F and EG1-H, 373 residues from EG1-T and 434 residues from CBH1.

**Table 1.** Sequence identities calculated relative to the lengths of each one of the sequences (seq1 & seq2):

seq1\seq2	EG1-H	EG1-T	CBH1
10 EG1-F	58% & 58%	41% & 44%	41% & 38%
EG1-H	*****	41% & 44%	40% & 37%
EG1-T	*****	*****	47% & 40%

In Table 2 below is listed the disulfide bridges found in 15 EG1-F, EG1-H and CBH1. Based on structural homology the equivalent positions for EG1-T are indicated.

**Table 2.** Disulfide bridges in EG1-F, EG1-H and CBH1. Based on the sequence alignment in Fig. 1, the equivalent positions are indicated for EG1-T. The numbers refer to those in Fig. 5.

Res# (Fig. 5)	EG1-F	EG1-H	(EG1-T)	CBH1
4-75	*	*	*	4-72
19-25	18-24	18-24	19-25	19-25
25 52-74	48-70	51-73	47-68	50-71
64-70	60-66	63-69	58-64	61-67
148-416	138-365	140-365	137-336	138-397
182-220	172-195	172-195	169-192	172-210
186-219	176-194	176-194	173-191	176-209
30 240-266	215-234	215-234	212-?	230-256
248-253	223-228	223-228	217-222	238-243
271-350	239-315	239-315	?-291	261-331

EG1-H and EG1-F contain the same 9 disulfide bridges.

The deletion around Res#260 in EG1-T prevents the formation of two disulfide bridges: C215-C234 & C239-C315 (EG1-H numbering). It is possible that the EG1-T structure has moved relative to EG1-H and EG1-F to enable the formation of a disulfide bridge between C215 and C315 (C212 & C291 in EG1-T numbering). The remaining 7 disulfide bridges found in EG1-F and EG1-H are also present in EG1-T.

10

CBH1 contains the same 9 disulfide bridges as EG1-F and EG1-H, and one additional disulfide bridge in the N-terminal region.

15 CBH1 contains the most insertions relative to the others, and these insertions are predominantly located at the edges of the substrate binding cleft, possibly contributing to the fact that CBH1 is a cellobiohydrolase.

20 When attempting to explain the improved alkaline activity of EG1-H and EG1-F relative to EG1-T, the following loops may be the most interesting, as they contain charges in the proximity of the active site. Charges can alter the pKa values of the catalytic residues, and interact with the transfer of 25 electrons during catalysis.

a) the 11-residue insertions (relative to EG1-T) at pos. 229 in EG1-H and EG1-F (Res#254) are located above the active site residues (E197, D199 & E202) and contain 2 or 3 charged 30 residues.

b) the 5-residue insertions (relative to EG1-T) at pos. 320 in EG1-H and EG1-F (Res#355) are located at the end of the substrate binding pocket and contain 2 or 3 charged 35 residues.

c) the 2-residue insertions (relative to EG1-T) at pos. 259 in EG1-H and EG1-F (Res#291) are located close to the b) insertion above, and contain a charged lysine residue.

5 The amino acid compositions of the four enzymes, i.e. EG1-F, EG1-H, EG1-T and CBH1) including the residues used in the sequence identity Table 1 above (i.e. including Res#453), are shown in Table 3.

10 Table 3. Amino acid compositions & pKa values used for pI calculation

		EG1-F	EG1-H	EG1-T	CBH1	pKa
15	Asp	21	18	19	24	3.5
	Asn	28	22	34	30	—
	Thr	22	28	34	46	—
	Ser	26	17	46	50	—
	Glu	20	31	10	19	4.0
20	Gln	18	12	16	18	—
	Pro	17	24	18	19	—
	Gly	44	47	43	51	—
	Ala	31	20	22	27	—
	Val	21	24	19	21	—
25	Cys	18	18	16	20	9.3
	Met	11	11	9	6	—
	Ile	22	14	11	10	—
	Leu	20	25	23	24	—
	Tyr	16	19	19	20	9.9, 11.6 or 12.5
	Phe	9	15	8	15	—

<b>Lys</b>	31	23	9	13	10.0
<b>His</b>	8	10	5	4	6.4
<b>Trp</b>	6	7	6	9	-
<b>Arg</b>	13	17	6	7	12.8

5 Assumed pKa's for Tyr 50% pH 9.9, 25% pH 11.6 & 25% Ph 12.5.

The isoelectric points (pI) were estimated for each of the four cellulases (shown in Table 4 below), employing standard pKa values. It was assumed that the N-terminals are blocked,  
 10 that no free C-terminal is present in the core enzymes, and that no metal ions are bound. The calculations do not consider the effects of deaminations.

**Table 4. Isoelectric point**

15

	<b>pI calculated</b>	<b>pI found</b>
<b>EG1-F</b>	8.9	about 9
<b>EG1-H</b>	5.7	about 5
<b>EG1-T</b>	4.0	
<b>CBH1</b>	3.8	

20

The difference between the calculated and actual pI values may be due to deaminating, e.g. Asn to Asp, which lower the actual pI.

25 From the amino acid compositions and the pKa values it is possible to calculate at different pH values the (partial) charges on all titrable amino acids. In this way the net charges and the sum of positive and negative charges were calculated at pH 4, 6, 8 and 10, as shown in Table 5.

30

The two alkaline cellulases, EG1-H and EG1-F, share the common characteristic, that over a broad pH-interval (pH 4-10)

they contain at least 70 charged residues. The acidic EG1-T in contrast contains fewer than 50 charged residues within this pH-interval. The more densely charged surfaces of EG1-F and EG1-H may be responsible for the improved performance 5 in laundry detergents relative to EG1-T.

**Table 5.** Charges as a function of pH. The first number is the net charge (e.g.  $(+13)+(-17)=-4$ ), the second is the total number of charges 10 (e.g.  $|+13|+|-17|=+30$ ).

	EG1-F	EG1-H	EG1-T	CBH1
pH 4	+26 / 78	+21 / 79	+1 / 39	-4 / 52
pH 6	+9 / 90	-1 / 96	-10 / 47	-20 / 66
pH 8	+3 / 85	-9 / 89	-14 / 44	-23 / 63
15 pH 10	-17 / 74	-26 / 83	-24 / 45	-35 / 62

To examine the active site region in more detail, amino acids located within 10 Å of the active site residues E197, D199 and E202 were identified for EG1-H and EG1-F. The following residues belong to this 10 Å subset in EG1-H (EG1-H numbering):

108, 124, 129, 131, 133, 135, 138-139, 141-151, 171-177, 193-220, 233-243, 245, 252, 266, 268, 270, 272, 280, 283, 285, 287, 310, 323, 326, 328, 331-336, 338-347, 354, 356-358, 362, 384, 386.

25

The 10 Å subset in EG1-F contain essentially the same residues as EG1-H 10 Å subset. EG1-T differs more significantly, in particular with respect to the segments around 219-221 and 230-240 in EG1-H and EG1-F, which are absent in EG1-30 T. Approx. 80 % sequence identity exists between EG1-F and EG1-H within the 10 Å subset, whereas the residues in the equivalent 10 Å subset for EG1-t are more different. Of particular interest are differences involving charges.

Within the 10 Å subset a number of mutations in EG1-H & EG1-F, aiming at affecting catalysis by changing electrostatics, are contemplated based on sequence homology to EG1-T. To decrease pI within this region M142E, K217A, K218T (in EG1-H 5 only) and R245G is contemplated, and to increase pI E150Q, I310D, E334K (in EG1-H) and D334K (in EG1-F) are contemplated.

The following amino acid residues are in close contact with 10 cellobiose (bound to the EG1-F); the numbering refers to the EG1-F numbering:

Hydrogen bonding between enzyme and inhibitor:

- a) R106 conserved in all 4 cellulases
- 15 b) Y145 conserved in all 4 cellulases
- c) S345 conserved in all 4 cellulases

Within 5 Å of cellubiose (excluding three active site residues, and the three H-bonding residues above):

- 20 d) D34 conserved in all 4 cellulases
- e) W51 also W51 in EG1-H. This W appears to be important for binding of the second sugar moiety in cellubiose (relative to the active site).
- 25 f) S104 conserved in all EG1s
- g) A143 also A143 in EG1-H
- h) Y171 conserved in all 4 cellulases
- i) D173 conserved in all 4 cellulases
- 30 j) Q175 conserved in all 4 cellulases
- k) Y177 not conserved
- l) W347 conserved in all 4 cellulases. This W347 appears to be important for binding of the first sugar moiety in cellobiose (relative to the active site).

35

Most of these residues are highly conserved. This implies that mutating them may be not of any advantage, but it cer-

tainly does not mean that the performance of EG1s cannot be improved by replacement of these residues. They are all located in the active site region, and in fact this makes them very interesting, as property changes are likely to be more significant. Therefore, it is contemplated that substitutions at all these positions are preferred substitutions.

The advantage of EG1-H is its ability to induce soil removal with minimal fabric damage. What is characteristic about EG1-H and EG1-F compared to Carezyme (4egv.pdb) and *Thermomonospora fusca* EG1 is a comparatively deep substrate binding cleft, possibly preventing the access of intact cotton fibers into the catalytic site.

In EG1-F and EG1-H the substrate binding cleft is 18-20 Å deep when measuring the distances between the Cα atoms of the active site residues (E197, D199 & E202) and the Cα atoms of the residues located at the upper rim of the substrate binding pocket (G351 & A229 in EG1-H). The pocket is approx 19 Å wide, when measuring between Cα atoms of the two rim residues.

In contrast, the depth of the substrate binding pocket in Carezyme (4egv.pdb), when measured in a similar manner, is only 8-10 Å, whereas the width at the rim is approx. 9 Å.

In *T. fusca* EG1 (1tml.pdb) the depth of the pocket is approx. 10 Å, and the width approx. 18 Å.

The present invention relates to a cellulase which is selected from the group consisting of cellulases classified in family 7 as described in Henrissat, B. et al.: Biochem. J. (1993), 293, p. 781-788, and cellulase variants derived from a parent cellulase classified in family C, wherein the cellulase comprises a core and optionally a C-terminal link consisting of 10 amino acids at the most, provided that the

cellulase is different from the endoglucanase having the amino acid sequence listed in Figure 1.

The classification by Henrissat is a new classification system for glycosyl hydrolases based on sequence comparisons and hydrophobic cluster analyses which have shown that the catalytic domains of glycosyl hydrolases fall into 45 distinct families, of which 11 (originally denoted A-K) contain enzymes with cellulolytic activity.

10

Thus far, structures of the catalytic domains of cellulases from four families have been published:

Cellobiohydrolase II (CBH II) from *Trichoderma reesei* (Rouvinen et al. 1990) and endocellulase E2 from *Thermomonospora fusca* (Sapezio et al. 1993) from family 6(B),  
Cellobiohydrolase I (CBH I) from *T. reesei* (Divne et al. 1994) from family 7 (C),  
Cela from *Clostridium thermocellum* (Juy et al. 1992) from family 9(E); and the endoglucanase V from *H. insolens* (Davies et al. 1993) from family 45(K).

The cellulases of the invention may be obtainable by or derived from a strain of *Humicola*, *Trichoderma*, *Myceliophthora*, *Penicillium*, *Irpex*, *Aspergillus*, *Scytalidium* or *Fusarium*,  
25 preferably from a strain of *Humicola*, *Trichoderma*, *Myceliophthora*, *Scytalidium* or *Fusarium*, more preferably from a strain of *Humicola insolens*, *Fusarium oxysporum*, *Myceliophthora thermophila* or *Trichoderma reesei*.

30 The cellulase of the invention can advantageously have one or more insertions of between 1 and 25, preferably between 1 and 20, amino acid residues; preferably the insertion is relative to EG1-Tat position 230-240 in EG1-H and EG1-F and part of which is located within 10Å of the active site residues E197, D199 and E202.



In another aspect, the cellulase of the invention has a substrate binding cleft with a depth of at least 12Å, preferably 15Å, when measuring between Cα atoms of the active site residues located at the bottom of the cleft and Cα atoms of residues located at the rim of the substrate binding cleft immediately above the active site residues.

According to the invention, cellulases which essentially consists of the core and optionally a C-terminal link having 10 10 amino acids at the most may perform excellent for particulate soil removal when used for washing/laundry or fabric softening purposes. In Figure 1 is disclosed the amino acid sequence of a genetically truncated endoglucanase from *Humicola insolens* of 402 amino acid residues which is disclosed 15 in WO 91/17244 as mentioned above (denoted EG I'). In Figure 3 is disclosed the amino acid sequence of a genetically truncated endoglucanase from *Humicola insolens* of 402 amino acid residues, this particular endoglucanase hereinafter being denoted EG I\*. In Figure 4 is disclosed the amino acid 20 sequence of a genetically truncated endoglucanase from *Fusarium oxysporum*, this particular endoglucanase hereinafter being denoted EGI-Fus. In figure 6 is disclose the amino acid sequence of an endoglucanase from *Myceliophthora thermophila*. Without being bound to the theory it is believed 25 that an enhanced enzyme activity may be obtained by providing cellulases, especially endoglucanases, which essentially consists of a core. The cellulase may further comprise a C-terminal link, a "tail", which is relatively short, the short C-terminal link not contributing negatively to the 30 enzyme activity. Thus it is believed that cellulases of the invention may be derived from known cellulases e.g. by "truncating" the C-terminal wholly or partly from the enzyme protein in question. The mentioned EG I\* is thus derived from the known endoglucanase EG I (see WO 91/17244, Fig.14A- 35 E) by eliminating the last 13 amino acids of the coding region. Furthermore, the amino acid residues in the positions 162, 203, 211 and 401, respectively, are substituted (162:

Ser to Pro; 203: Val to Ala; 211: Val to Ala; 401: Val to Leu).

It is contemplated that cellulases which have an amino acid sequence being at least 60% homologous with the amino acid sequences listed in Figure 1, 3 and 4, respectively, also have enhanced activity resulting in improved soil removal from fabrics.

10 In Figure 2 is listed an alignment of the amino acid sequence of three known endoglucanases from *Humicola insolens* (denoted EGIHUM and having 415 amino acids), *Fusarium oxysporum* (denoted Cendofus and having 409 amino acids) and *Trichoderma reesei* (denoted Egltrite and having 435 amino acids), respectively.

Accordingly, the invention further relates to a cellulase which is derivable from a strain of *Humicola insolens* and comprises the amino acid residues 1-397 listed in Figure 3  
20 and optionally a C-terminal link consisting of 10 amino acids at the most, or a variant of said cellulase having an amino acid sequence being at least 60% homologous with said sequence; a cellulase which is derivable from a strain of *Fusarium oxysporum* and comprises the amino acid residues 1-  
25 401 listed in Figure 4 and optionally a C-terminal link consisting of 10 amino acids at the most, or a variant of said cellulase having an amino acid sequence being at least 60% homologous with said sequence; a cellulase which is derivable from a strain of *Trichoderma reesei* and comprises the  
30 amino acid residues 1-369 listed in Figure 2 (the amino acid sequence denoted Egltrire) and optionally a C-terminal link consisting of 10 amino acids at the most, or a variant of said cellulase having an amino acid sequence being at least 60% homologous with said sequence; and cellulase according  
35 to claim 4, which is derivable from a strain of *Myceliophthora thermophila* and comprises the amino acid residues 1-456, preferably the residues 1-420, more preferably the re-

sidues 21-420, listed in Figure 6 and optionally a C-terminal link consisting of 10 amino acids at the most, or a variant of said cellulase having an amino acid sequence being at least 60% homologous with said sequence, provided that  
5 the cellulase is different from the endoglucanase having the amino acid sequence listed in Figure 1. The pI of the cellulase from *Myceliophthora thermophila* was found to be 4.0.

Examples of variants of these cellulases are variants  
10 wherein one or more of the following amino acid residues are substituted: N89Q, N89Q+N247Q, H123N, T385N, Q399N, E202A, S37W+P39W.

Other useful variants are those wherein one or more of the  
15 following amino acid residues are substituted: M142E, K217A, K217A+K218T, R245G, I310D, E150Q, E334K, M198L.

Yet other useful cellulase variant are those wherein one or more of the following amino acid residues within 5Å of bound  
20 cellobiose are substituted:

R106x, Y145x, S345x, D34x, W51x, S104x, A143x, Y171x, D173x, Q175x, Y177x, W347x, where x is chosen to modify H-bonding potential and/or hydrophobic interaction with the substrate.  
25

The activity of the present cellulases with respect to soil removal may be correlated to specific analytical methods.

Accordingly, the present invention further relates to a cellulase having high activity on cellotriose in the presence  
30 of a detergent matrix, high dispersing action on carbon black, and high alkaline activity on acid swollen cellulose at pH 8.

35 More specifically, the activity on cellotriose (see below) in the presence of a detergent matrix corresponds to an apparent  $k_{cat}$  at pH 8 of preferably at least 1 per sec; the

dispersing action on carbon black at pH 10 corresponds to a delta value preferably of at least 0.20 measured at 582 nm for 5 mg/l of cellulase (see e.g. example 2); and the alkaline activity on acid swollen cellulose at pH 8 corresponds to an apparent  $k_{cat}$  preferably of at least 10 per sec (see below).

EG I\* (from *Humicola insolens*; see Fig. 3) has an apparent molecular weight (MW) of about 50kD due to glycosylation of the molecule. It is believed that the "true" MW is about 46kD. The pI of EG I\* is at least about 0.4 lower than of EG I, since pI of EG I\* is about 5.1-5.3 whereas pI of EG I is about 5.5-6.2.

EGI-Fus (from *Fusarium oxysporum*; see Fig. 4) has a apparent molecular weight (MW) of 48 kD; the amino acid composition gives 45 kD with 2 N glycosylation sites. The actual pI is above 9, and the theoretical pI is 9 which has been calculated based on the amino acid composition and using the pKa values from C. Tanford in Adv. Protein Chem. vol 17 pages 69-165, 1962. The molar extinction coefficient (based on the amino acid composition) has been calculated to 58180. It has been found that the stability of EGI-Fus is optimal at 50 degrees Celsius. The enzyme exhibits no activity above 60 degrees Celsius. The catalytic activity on cellotriose at pH 8,5 and 40°C has been calculated to 5,5  $K_{cat}$  per sec.  $K_m$  is 0,5 mM. Further, the activity on CMC is about 315 ECU per mg protein. The activity of EGI-Fus can be inactivated by 3-epoxybutyl cellobioside; see e.g. G. Legler and E. Bause, Carbohydrate Research vol 28 (1973) page 45-52: Epoxyalkyl oligo (1-4) beta-D-Glucosides as active site directed inhibitors of cellulases.

The cellulases of the invention may be obtained from the microorganism in question by use of any suitable technique. For instance, a cellulase preparation may be obtained by fermentation of a microorganism and subsequent isolation of

a cellulase containing preparation from the fermented broth or microorganism by methods known in the art, but more preferably by use of recombinant DNA techniques as known in the art. Such method normally comprises cultivation of a host cell transformed with a recombinant DNA vector capable of expressing and carrying a DNA sequence encoding the cellulase component in question, in a culture medium under conditions permitting the expression of the enzyme and recovering the enzyme from the culture.

10

Cloning a DNA sequence encoding a cellulase

The DNA sequence encoding a parent cellulase may be isolated from any cell or microorganism producing the cellulase in question by various methods, well known in the art. First a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the cellulase to be studied. Then, if the amino acid sequence of the cellulase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify cellulase-encoding clones from a genomic library of bacterial DNA, or from a fungal cDNA library. Alternatively, a labelled oligonucleotide probe containing sequences homologous to cellulase from another strain of bacteria or fungus could be used as a probe to identify cellulase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying cellulase-producing clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming cellulase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for cellulase. Those bacteria containing cellulase-bearing plasmid will produce colonies surrounded by a halo of clear agar, due to digestion of the substrate by secreted cellulase.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and 5 M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., The EMBO J. 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in 10 appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic 15 or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA sequence, in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Sai- 20 ki et al., Science 239, 1988, pp. 487-491.

#### Expression of cellulase variants

According to the invention, a mutated cellulase-coding sequence 25 produced by methods described above, or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a re- 30 pressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a "signal sequence" may be inserted prior to the cellulase-coding sequence. For expression under the direction of control sequences, a target gene to be treated according to the 35 invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can support the

transcription of the mutant cellulase gene, include but are not limited to the prokaryotic  $\beta$ -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, 5 Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94.

According to one embodiment B. subtilis is transformed by an 10 expression vector carrying the mutated DNA. If expression is to take place in a secreting microorganism such as B. subtilis a signal sequence may follow the translation initiation signal and precede the DNA sequence of interest. The signal sequence acts to transport the expression product to the 15 cell wall where it is cleaved from the product upon secretion. The term "control sequences" as defined above is intended to include a signal sequence, when is present.

In a currently preferred method of producing cellulase variants of the invention, a filamentous fungus is used as the host organism. The filamentous fungus host organism may conveniently be one which has previously been used as a host for producing recombinant proteins, e.g. a strain of Aspergillus sp., such as A. niger, A. nidulans or A. oryzae. The 25 use of A. oryzae in the production of recombinant proteins is extensively described in, e.g. EP 238 023.

For expression of cellulase variants in Aspergillus, the DNA sequence coding for the cellulase variant is preceded by a 30 promoter. The promoter may be any DNA sequence exhibiting a strong transcriptional activity in Aspergillus and may be derived from a gene encoding an extracellular or intracellular protein such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.

35

Examples of suitable promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei

aspartic proteinase, A. niger neutral  $\alpha$ -amylase, A. niger acid stable  $\alpha$ -amylase, A. niger glucoamylase, Rhizomucor miehei lipase, A. oryzae alkaline protease or A. oryzae triose phosphate isomerase.

5

In particular when the host organism is A. oryzae, a preferred promoter for use in the process of the present invention is the A. oryzae TAKA amylase promoter as it exhibits a strong transcriptional activity in A. oryzae. The sequence 10 of the TAKA amylase promoter appears from EP 238 023.

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

15 The techniques used to transform a fungal host cell may suitably be as described in EP 238 023.

To ensure secretion of the cellulase variant from the host cell, the DNA sequence encoding the cellulase variant may be 20 preceded by a signal sequence which may be a naturally occurring signal sequence or a functional part thereof or a synthetic sequence providing secretion of the protein from the cell. In particular, the signal sequence may be derived from a gene encoding an Aspergillus sp. amylase or glucoamylase, a gene encoding a Rhizomucor miehei lipase or protease, 25 or a gene encoding a Humicola cellulase, xylanase or lipase. The signal sequence is preferably derived from the gene encoding A. oryzae TAKA amylase, A. niger neutral  $\alpha$ -amylase, A. niger acid-stable  $\alpha$ -amylase or A. niger gluco- 30 amylase.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing Aspergillus cells. The transformants are usually stable and may be cul- 35 tured in the absence of selection pressure. However, if the transformants are found to be unstable, a selection marker introduced into the cells may be used for selection.



The mature cellulase protein secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

For example, EGI-Fus (from *Fusarium oxysporum*, Fig. 4) was produced by *Aspergillus oryzae* after transformation with a plasmid containing the disclosed sequence and using the normal taka promotor and AMG terminator. Fermentation of the transformed *A. oryzae* strain gave a yield of 380 ECU per ml. The fermentation broth was purified by filtration and concentration using ultrafiltration. The concentrate was adjusted to pH 5 and applied to a high performance S-Sepharose column equilibrated with 50 mM sodium acetate, pH 5.0. The enzyme bound and was eluted with a sodium chloride salt gradient. The pure endoglucanase (EGI-Fus) was eluted with 0.5 M sodium chloride.

#### Detergent Compositions

According to the invention, the cellulase of the invention or an endoglucanase derived from a strain of *Humicola insolens* and having the amino acid sequence listed in Figure 1 and an apparent molecular weight of about 50 kD measured in SDS-PAGE may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000, ethoxylated nonyl-

phenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216. Accordingly, the present invention further relates to a detergent additive comprising a cellulase of the present invention or an endoglucanase derived from a strain of *Humicola insolens* and having the amino acid sequence listed in Figure 3 and an apparent molecular weight of about 50 kD measured in SDS-PAGE.

20 The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

25 The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonyl-phenol ethoxylate, alkylpolyglycoside, alkyl dimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid

monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or 5 more other enzymes, such as amylase, lipase, cutinase, protease, other cellulases, peroxidase, and oxidase, e.g., lacase).

The detergent may contain 1-65% of a detergent builder or 10 complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). 15 The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) 20 (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise 25 a  $H_2O_2$  source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetythylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone 30 type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar 35 or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the

composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

10 The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of detergent compositions within the scope 15 of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	7	-	12%
5	Alcohol ethoxysulfate (e.g. C <sub>12-18</sub> alcohol, 1-2 EO) or alkyl sulfate (e.g. C <sub>16-18</sub> )	1	-	4%
	Alcohol ethoxylate (e.g. C <sub>14-15</sub> alcohol, 7 EO)	5	-	9%
10	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	14	-	20%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	2	-	6%
	Zeolite (as NaAlSiO <sub>4</sub> )	15	-	22%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	-	6%
15	Sodium citrate/citric acid (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> /C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> )	0	-	15%
	Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	11	-	18%
	TAED	2	-	6%
	Carboxymethylcellulose	0	-	2%
20	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0	-	3%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
25	Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0	-	5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
5	Alcohol ethoxysulfate (e.g. C <sub>12-18</sub> alcohol, 1-2 EO or alkyl sulfate (e.g. C <sub>16-18</sub> ))	1 - 3%
	Alcohol ethoxylate (e.g. C <sub>14-15</sub> alcohol, 7 EO)	5 - 9%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	15 - 21%
10	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1 - 4%
	Zeolite (as NaAlSiO <sub>4</sub> )	24 - 34%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	4 - 10%
	Sodium citrate/citric acid (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> /C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> )	0 - 15%
15	Carboxymethylcellulose	0 - 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
20	Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	5	-	9%
	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO)	7	-	14%
5	Soap as fatty acid (e.g. C <sub>16-22</sub> fatty acid)	1	-	3%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	10	-	17%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	3	-	9%
	Zeolite (as NaAlSiO <sub>4</sub> )	23	-	33%
10	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	-	4%
	Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	8	-	16%
	TAED	2	-	8%
	Phosphonate (e.g. EDTMPA)	0	-	1%
	Carboxymethylcellulose	0	-	2%
15	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0	-	3%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
20	Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0	-	5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	8	- 12%
	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO)	10	- 25%
5	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	14	- 22%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1	- 5%
	Zeolite (as NaAlSiO <sub>4</sub> )	25	- 35%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	- 10%
	Carboxymethylcellulose	0	- 2%
10	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1	- 3%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
15	Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

## 5) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
20	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO or C <sub>12-15</sub> alcohol, 5 EO)	12	- 18%
	Soap as fatty acid (e.g. oleic acid)	3	- 13%
25	Alkenylsuccinic acid (C <sub>12-14</sub> )	0	- 13%
	Aminoethanol	8	- 18%
	Citric acid	2	- 8%
	Phosphonate	0	- 3%
	Polymers (e.g. PVP, PEG)	0	- 3%
30	Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
	Ethanol	0	- 3%
	Propylene glycol	8	- 14%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
35	Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0	- 5%



6) An aqueous structured liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
5	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	3	- 9%
	Soap as fatty acid (e.g. oleic acid)	3	- 10%
10	Zeolite (as NaAlSiO <sub>4</sub> )	14	- 22%
	Potassium citrate	9	- 18%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. PEG, PVP)	0	- 3%
15	Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0	- 3%
	Glycerol	0	- 5%
20	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0	- 5%

25

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Fatty alcohol sulfate	5	- 10%
30	Ethoxylated fatty acid monoethanol-amide	3	- 9%
	Soap as fatty acid	0	- 3%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	5	- 10%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1	- 4%
	Zeolite (as NaAlSiO <sub>4</sub> )	20	- 40%
35	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	2	- 8%
	Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	12	- 18%
	TAED	2	- 7%

	Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
5	Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0	-	5%

8) A detergent composition formulated as a granulate comprising

	Linear alkylbenzenesulfonate (calculated as acid)	8	-	14%
	Ethoxylated fatty acid monoethanolamide	5	-	11%
15	Soap as fatty acid	0	-	3%
	Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	4	-	10%
	Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$ )	1	-	4%
	Zeolite (as $\text{NaAlSiO}_4$ )	30	-	50%
	Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	3	-	11%
20	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ )	5	-	12%
	Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
25	Minor ingredients (e.g. suds suppressors, perfume)	0	-	5%

9) A detergent composition formulated as a granulate comprising

	Linear alkylbenzenesulfonate (calculated as acid)	6	- 12%
5	Nonionic surfactant	1	- 4%
	Soap as fatty acid	2	- 6%
	Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	14	- 22%
	Zeolite (as $\text{NaAlSiO}_4$ )	18	- 32%
	Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	5	- 20%
10	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ )	3	- 8%
	Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$ )	4	- 9%
	Bleach activator (e.g. NOBS or TAED)	1	- 5%
	Carboxymethylcellulose	0	- 2%
15	Polymers (e.g. polycarboxylate or PEG)	1	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
20	Minor ingredients (e.g. optical brightener, perfume)	0	- 5%

10) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	- 23%
5	Alcohol ethoxysulfate (e.g. C <sub>12-15</sub> alcohol, 2-3 EO)	8	- 15%
	Alcohol ethoxylate (e.g. C <sub>12-15</sub> al- cohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	3	- 9%
10	Soap as fatty acid (e.g. lauric acid)	0	- 3%
	Aminoethanol	1	- 5%
	Sodium citrate	5	- 10%
	Hydrotrope (e.g. sodium toluensulfonate)	2	- 6%
15	Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
	Carboxymethylcellulose	0	- 1%
	Ethanol	1	- 3%
	Propylene glycol	2	- 5%
20	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0	- 5%

11) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	20	- 32%
5	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	6	- 12%
	Aminoethanol	2	- 6%
	Citric acid	8	- 14%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	1	- 3%
10	Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0	- 3%
15	Glycerol	3	- 8%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
20	Minor ingredients (e.g. hydro-tropes, dispersants, perfume, optical brighteners)	0	- 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

25	Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25	- 40%
30	Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 10%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	8	- 25%
	Soluble silicates (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	5	- 15%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	- 5%
	Zeolite (as NaAlSiO <sub>4</sub> )	15	- 28%
35	Sodium perborate (as NaBO <sub>3</sub> ·4H <sub>2</sub> O)	0	- 20%
	Bleach activator (TAED or NOBS)	0	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
40	Minor ingredients (e.g. perfume, optical brighteners)	0	- 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C<sub>12</sub>-C<sub>18</sub>) alkyl sulfate.

5 14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	(C <sub>12</sub> -C <sub>18</sub> ) alkyl sulfate	9	- 15%
	Alcohol ethoxylate	3	- 6%
	Polyhydroxy alkyl fatty acid amide	1	- 5%
10	Zeolite (as NaAlSiO <sub>4</sub> )	10	- 20%
	Layered disilicate (e.g. SK56 from Hoechst)	10	- 20%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	3	- 12%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	0	- 6%
15	Sodium citrate	4	- 8%
	Sodium percarbonate	13	- 22%
	TAED	3	- 8%
	Polymers (e.g. polycarboxylates and PVP=	0	- 5%
20	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0	- 5%

25

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	(C <sub>12</sub> -C <sub>18</sub> ) alkyl sulfate	4	- 8%
	Alcohol ethoxylate	11	- 15%
5	Soap	1	- 4%
	Zeolite MAP or zeolite A	35	- 45%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	2	- 8%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	0	- 4%
	Sodium percarbonate	13	- 22%
10	TAED	1	- 8%
	Carboxymethyl cellulose	0	- 3%
	Polymers (e.g. polycarboxylates and PVP)	0	- 3%
15	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0	- 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system

(e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The endoglucanase of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the endoglucanase may be added in an amount corresponding to 0.00001-1 mg (calculated as pure enzyme protein) of endoglucanase per liter of wash liquor.

10

In yet another aspect, the present endoglucanases may be used in fabric softeners, e.g. as described in Surfactant and Consumer Products, Ed. by J. Falbe, 1987, pp 295-296; Tenside Surfactants Detergents, 30 (1993), 6, pp 394-399; 15 JAOCS, Vol. 61 (1984), 2, pp 367-376; EP 517 762; EP 123 400; WO 92/19714; WO 93/19147; US 5,082,578; EP 494 769; EP 544 493; EP 543 562; US 5,235,082; EP 568 297; EP 570 237.

The present invention also relates to a washing process 20 wherein soiled fabric is treated with a cellulase of the invention or an endoglucanase derived from a strain of *Humicola insolens* and having the amino acid sequence listed in Figure 3 and an apparent molecular weight of about 50 kD measured in SDS-PAGE.

25

It is contemplated that, dependent on the specificity of the modified cellulase, it may be employed for one or possibly more of the applications mentioned above, i.e. in the baking industry, in the wine and juice industry, for animal feed, 30 and in textile and papermaking pulp processing. In a particular embodiment, the enzyme preparation of the invention may comprise a combination of one or more modified cellulases with enzymes selected from the group consisting of unmodified or modified amylases, lipases, proteases, oxidore- 35 ductases and hemicellulases.



### Pulp and paper applications

In the papermaking pulp industry, the cellulase and/or enzyme preparation according to the invention may be applied 5 advantageously e.g. as follows:

- For debarking: pretreatment with the cellulase and/or enzyme preparation according to the invention may degrade the cambium layer prior to debarking in mechanical drums resulting in advantageous energy savings. 10

- For defibration: treatment of a material containing cellulosic fibers with the cellulase and/or enzyme preparation of the invention prior to refining or beating may result in 15 reduction of the energy consumption due to the hydrolysing effect of the cellulase on the interfibre surfaces. Use of the cellulase and/or enzyme preparation of the invention may result in improved energy savings as compared to the use of unmodified enzymes, since it is believed that the modified 20 cellulase may possess a higher ability to penetrate fibre walls.

- For fibre modification, i.e. improvement of fibre properties where partial hydrolysis across the fibre wall is needed which requires deeper penetrating enzymes (e.g. in order 25 to make coarse fibers more flexible). Deep treatment of fibers has so far not been possible for high yield pulps e.g. mechanical pulps or mixtures of recycled pulps. This has been ascribed to the nature of the fibre wall structure 30 that prevents the passage of enzyme molecules due to physical restriction of the pore matrix of the fibre wall. It is contemplated that the modified (i.e. derivatised) cellulases of the invention are capable of penetrating into the fibre wall.

35

- For drainage improvement. The drainability of papermaking pulps may be improved by treatment of the pulp with hydroly-

sing enzymes, e.g. cellulases. Use of the modified cellulase and/or enzyme preparation according to the invention may be more effective, e.g. result in a higher degree of loosening bundles of strongly hydrated micro-fibrils in the fines 5 fraction (consisting of fibre debris) that limits the rate of drainage by blocking hollow spaces between fibers and in the wire mesh of the paper machine. The Canadian standard freeness (CSF) increases and the Schopper-Riegler drainage index decreases when pulp is subjected to cellulase treat- 10 ment, see e.g. US patent 4,923,565; TAPPI T227, SCAN C19:65 which are hereby incorporated by reference.

- For inter fibre bonding. Hydrolytic enzymes are applied in the manufacture of papermaking pulps for improving the inter 15 fibre bonding. The enzymes rinse the fibre surfaces for impurities e.g. cellulosic debris, thus enhancing the area of exposed cellulose with attachment to the fibre wall, thus improving the fibre-to-fibre hydrogen binding capacity. This process is also referred to as dehornification. Paper and 20 board produced with a cellulase containing enzyme preparation according to the invention may have an improved strength or a reduced grammage, a smoother surface and an improved printability. These improvements are believed to be a result of the improved penetrability of the modified/der- 25 ivatised enzyme(s).

- For enzymatic deinking. Partial hydrolysis of recycled paper during or upon pulping by use of hydrolysing enzymes such as cellulases are known to facilitate the removal and 30 agglomeration of ink particles. Use of a modified cellulase and/or enzyme preparation according to the invention may give a more effective loosening of ink from the surface structure due to a better penetration of the enzyme molecules into the fibrillar matrix of the fibre wall, thus soft- 35 ning the surface whereby ink particles are effectively loosened. The agglomeration of loosened ink particles are also improved, due to a more efficient hydrolysis of cellulosic

fragments found attached to ink particles originating from the fibres.

The treatment of lignocellulosic pulp may, e.g., be performed as described in WO 91/14819, WO 91/14822, WO 92/17573 and WO 92/18688.

#### Textile applications

10

In another embodiment, the present invention relates to use of the modified cellulase and/or enzyme preparation according to the invention in the bio-polishing process. Bio-Polishing is a specific treatment of the yarn surface which improves fabric quality with respect to handle and appearance without loss of fabric wettability. The most important effects of Bio-Polishing can be characterized by less fuzz and pilling, increased gloss/luster, improved fabric handle, increased durable softness and altered water absorbency.

20 Bio-Polishing usually takes place in the wet processing of the manufacture of knitted and woven fabrics. Wet processing comprises such steps as e.g. desizing, scouring, bleaching, washing, dying/printing and finishing. During each of these steps, the fabric is more or less subjected to mechanical action. In general, after the textiles have been knitted or woven, the fabric proceeds to a desizing stage, followed by a scouring stage, etc. Desizing is the act of removing size from textiles. Prior to weaving on mechanical looms, warp yarns are often coated with size starch or starch derivatives in order to increase their tensile strength. After weaving, the size coating must be removed before further processing the fabric in order to ensure a homogeneous and wash-proof result. It is known that in order to achieve the effects of Bio-Polishing, a combination of cellulolytic and mechanical action is required. It is also known that "super-softness" is achievable when the treatment with cellulase is combined with a conventional treatment

with softening agents. It is contemplated that use of the modified cellulase and/or enzyme preparation of the invention for bio-polishing of cellulosic fabrics is advantageous, e.g. a more thorough polishing can be achieved. Bio-polishing may be obtained by applying the method described e.g. in WO 93/20278.

Degradation of plant material

- 10 In yet another embodiment, the present invention relates to use of a modified cellulase and/or enzyme preparation according to the invention for degradation of plant material e.g. cell walls.
- 15 It is contemplated that the modified cellulase and/or enzyme preparation of the invention is useful in the preparation of wine, fruit or vegetable juice in order to increase yield. Cellulases according to the invention may also be applied for enzymatic hydrolysis of various plant cell-wall derived
- 20 materials or waste materials, e.g. agricultural residues such as wheat-straw, corn cobs, whole corn plants, nut shells, grass, vegetable hulls, bean hulls, spent grains, sugar beet pulp, and the like. The plant material may be degraded in order to improve different kinds of processing,
- 25 facilitate purification or extraction of other components like purification of beta-glucan or beta-glucan oligomers from cereals, improve the feed value, decrease the water binding capacity, improve the degradability in waste water plants, improve the conversion of e.g. grass and corn to
- 30 ensilage, etc.

In a preferred embodiment of the invention, the cellulase is an endoglucanase. The cellulolytic activity of endoglucanase is determined relative to an analytical standard and may be expressed in the unit ECU.

5

Cellulolytic enzymes hydrolyse CMC, thereby decreasing the viscosity of the incubation mixture. The resulting reduction in viscosity may be determined by a vibration viscosimeter (e.g. MIVI 3000 from Sofraser, France).

10

Determination of the cellulolytic activity, measured in terms of ECU, may be determined according to the analysis method AF 301.1 which is available from the Applicant upon request.

15

The ECU assay quantifies the amount of catalytic activity present in the sample by measuring the ability of the sample to reduce the viscosity of a solution of carboxy-methylcellulose (CMC). The assay is carried out at 40°C, pH 7.5 using 20 a relative enzyme standard for reducing the viscosity of the CMC substrate.

#### Cellulase activity on cellotriose

25 The cellulase activity on cellotriose, in terms of  $k_{cat} \cdot s^{-1}$ , was determined by a coupled assay:

Cellotriose  $\rightarrow$  Glucose + Cellobiose (cat.: cellulase)

30 Glucose + O<sub>2</sub> + H<sub>2</sub>O  $\rightarrow$  Gluconate + H<sub>2</sub>O<sub>2</sub> (cat.: Glucoseoxidase)

H<sub>2</sub>O<sub>2</sub> + ABTS<sup>R</sup>  $\rightarrow$  ABTS<sup>Ox</sup> (cat.: Peroxidase)

which is followed spectrophotometrically at 418 nm (maximum 35 absorbance of ABTS<sup>Ox</sup> at 418 nm).

*Method:*

The GOD-Perid Test Kit (available from Boehringer Mannheim, art. 124 036) was used. The buffer-enzyme solution in the 5 test kit was dissolved in 500 ml milli Q water. pH of the solution was adjusted to 8.5 (NaOH).

80 mg of ABTS<sup>R</sup> (available from Boehringer Mannheim, art. 756 407) was dissolved in 10 ml GOD-Perid corresponding to a 10 total concentration of ABTS<sup>R</sup> of 10 mg/ml.

A substrate stock solution of 5 mmole (2.52 mg/ml) of cello-triose (available from Merck art. 24741) in water was prepared. Diluted solutions in water corresponding to 1000  $\mu$ -15 mole, 500  $\mu$ mole, 376  $\mu$ mole, 250  $\mu$ mole, 100 $\mu$ mole and 60  $\mu$ mole were prepared.

The reaction mixture was prepared by mixing 1 part of substrate solution with 1 part of GOD-Perid.

20

A solution of the cellulase enzyme to be determined in a concentration of 1.0 - 3.0  $\mu$ mole was prepared.

50  $\mu$ l of enzyme solution and 450  $\mu$ l of reaction mixture were 25 mixed.

The measurements were carried out on a HP 8452A Diode Array Spectrophotometer thermostated at 40°C, 1 cm cuvette, at a wavelength of 418 nm. The reaction was followed by measuring 30 the oxidation of ABTS every 20 sec for 600 sec in total.

*Calculations:*

The cellulase activity on cellotriase, in terms of  $k_{\text{cat}} \cdot \text{s}^{-1}$ , 35 was calculated from a Lineweaver-Burk plot (a plot of  $1/V$  versus  $1/[S]$ ): the slope and the intersection were determined by linear regression analysis.

The following constants were used for the calculations:

Cellulase:  $\epsilon = 66,310 \text{ M}^{-1} \cdot \text{cm}^{-1}$

ABTS<sup>OX</sup> :  $\epsilon = 0.0323 \text{ } \mu\text{mole}^{-1} \cdot \text{cm}^{-1}$

5

For EG I' from *Fusarium oxysporum* (Figure 4), the catalytic activity on cellotriose at pH 8,5 and 40°C was calculated to 5,5 K<sub>cat</sub> pr. sec. K<sub>m</sub> was 0.5 mM.

10

#### Determination of alkaline cellulase activity on amorphous cellulose

##### 15 Method:

Substrate preparation: 20 gram acid-swollen AVICEL<sup>®</sup> stock solution (see below for a preparation which can be stored for one month) was centrifuged for 20 min. at 5000 rpm., the  
20 supernatant was poured off, and the sediment was resuspended in 30 ml of buffer. Then centrifuged for 20 min. at 5000 rpm, the supernatant was poured off, and the sediment was resuspended in buffer to a total of 30 g.. This corresponds to a substrate concentration of 10 g AVICEL/litre.

25

Buffer: 0.1M Barbitol at pH 8.5 or 0.1M Glycine at pH 10.0

##### Enzyme solution:

The enzymes were diluted to an activity of 0.5 S-CEVU/ml at  
30 pH 8.5 or 0.75 S-CEVU/ml at pH 10.0.

##### Reagents:

2% NaOH, PHBAH-reagent: 1.5 g of p-hydroxy benzoic acid hy-  
drazide and 5.0 g sodium tartrate was dissolved in 100 ml of  
35 2% NaOH.

The substrate, the buffer and the enzyme solution were mixed as follows:

Substrate $\mu\text{l}$	Buffer $\mu\text{l}$	Enzyme sol. $\mu\text{l}$	Subst. conc. (final) g/l
50	1950	500	0.20
125	1875	500	0.50
250	1750	500	1.00
500	1500	500	2.00
750	1250	500	3.00
1000	1000	500	4.00

10

The substrate/buffer solution was preheated for 5 min at 40°C. Then the enzyme solution was added and the solution was whirlmixed for 5 sec., followed by incubation for 20 min. at 40°C.

15 The reaction was stopped by adding 500  $\mu\text{l}$  2% NaOH solution, followed by whirlmixing for 5 sec.

The samples were centrifuged for 20 min. at 5000 rpm.

1000  $\mu\text{l}$  of supernatant was transferred from the test tubes to new test tubes, and 500  $\mu\text{l}$  PHBAH-reagent was added, fol-

20 lowed by boiling for 10 min.

The test tubes were cooled in ice water.

The absorbance of the samples were measured on a spectrophotometer at 410 nm.

25 Standard glucose curve:

A stock solution containing 300 mg/l was diluted to 5, 10, 15 and 25 mg/l.

1000  $\mu\text{l}$  of the diluted standards were mixed with 500  $\mu\text{l}$  of  
30 PHBAH-reagent, and were treated as the other samples, see above.



#### Determination of activity.

The release of reducing glucose equivalent was calculated using the standard curve.

The enzyme concentration was calculated using the molar absorbance of 66310 ( $\epsilon$ ) for the EG I endoglucanase. The  $K_m$ ,  $V_{max}$  and  $K_{cat}$  was calculated from a Lineweaver-Burk plot using different substrate concentrations.

The molar absorbance of the cellulase variants having substituted tyrosines and tryptophanes was adjusted accordingly using a absorbance value for tryptophane of 5690( $\epsilon$ ) and for tyrosine of 1280( $\epsilon$ ) and cystein 120( $\epsilon$ ).

The extinction coefficients ( $\epsilon$ ) are disclosed in Gill, S.C. and Hippel, P.H.: Calculation of protein extinction coefficients from amino acid sequence data; Analytical Biochemistry vol 182, (319-326), (1989).

Each of the tested cellulases was purified to high homogeneity giving a single band in SDS-PAGE analysis (the ratio  $A_{280}/A_{260}$  was checked as being above 1.5).

#### Preparation of Acid swollen cellulose:

##### Materials:

- 25 5 g Avicel®. (Art. 2331 Merck)
- 150 ml 85% Ortho-phosphoric-acid. (Art. 573 Merck)
- 400 ml Acetone. (Art. 14 Merck)
- 1.3 l Deionized water (Milli Q)
- 1 l glass beaker
- 30 1 l glass filter funnel
- 2 l suction flask
- Ultra Turrax Homogenizer

##### Procedure:

- 35 The Acetone and the phosphoric-acid was cooled on ice.

The 5 g. Avicel® was moistened with water, then 150 ml of ice cold 85% Ortho-phosphoric-acid was added, and the mixture was placed on ice bath with weak stirring for 1 h.

100 ml of ice cold acetone was added with stirring, followed by transfer of the mixture to a glass filter funnel, followed by washing with 3 x 100 ml ice cold acetone and dry suction after each washing.

The filter cake was washed with 2 x 500 ml water and sucked as dry as possible after each wash.

10 The filter cake was resuspended to a total volume of 300 ml and blended to homogeneity (using the Ultra Turrax Homogenizer).

The resulting product was stored in a refrigerator.

15 The following result was obtained

EG I\* from *Humicola insolens* (Figure 3):

$k_{cat}$  at 8.5: 16 per sec

$k_{cat}$  at 10: 12 per sec

20

The extinction coefficient was 66310.

EGI-Fus from *Fusarium oxysporum* (Figure 4):

$k_{cat}$  at 8.5, 40°C: 16 per sec ( $k_m$  8g/l)

25  $k_{cat}$  at 10, 40°C: 4 per sec ( $k_m$  8g/l)

The molar extinction coefficient was 58180, calculated based on the amino acid composition.

30

#### EXAMPLE 1

Multi cycle terg-o-tometer test EG I\* versus EG I

35 The example illustrates the superior soil removal effects of EG I\* (truncated EG I, 402 amino acids, see Figure 3) versus EG I (415 amino acids, WO 91/17244 Fig. 14A-E). In the

example EMPA 101 swatches have been used as soil removal tracers (carbon black/olive oil).

The following detergent composition was used:

5		% by weight
	LAS, (Nansa 1169/p)	10.3
	AES, (Berol 452)	3.5
	SOAP (C18)	0.5
10	SOAP (C14)	0.5
	AEO (Dobanol 25-7)	6.4
	Sodiumxylenesulfonat	5.1
	Ethanol	0.7
	MPG	2.7
15	Glycerol	0.5
	Sodium sulphate	0.40
	Sodium carbonate	2.7
	Sodium citrate	4.4
	Citric acid	1.5
20	Water	Rest

#### Testing procedure

The test was based on a 2 cycle wash test in a terg-o-tome-25 ter using the detergent composition described above in a 0.3% solution with 1mM Ca++.

	Agitation	:	150 m/min
	Temperature	:	40°C
30	pH	:	8.2
	Swatches	:	EMPA 101 (2 swatches á 5X6 cm pr 100 ml)
	Washing time	:	20 minutes
	Rinse	:	10 minutes in tapwater
35	Drying	:	Line drying at room temperature
	Repetitions	:	2

Result

The soil removal result is given as Delta remmision R (Enzyme-treated versus blind) measured at 420 nm with an Elrepho apparatus (DataColor).

Enzyme		R (S.D.)
		Delta R
5	No enzyme	41.99 (0.41)
		0
10	EG I, 4 ECU/l	42.00 (0.28)
		0.01
	EG I, 8 ECU/l	41.24 (0.45)
		-0.75
15	EG I, 12 ECU/l	42.69 (0.32)
		0.71
	EG I, 20 ECU/l	43.05 (0.32)
		1.06
20	EG I*, 4 ECU/l	41.61 (0.37)
		-0.38
	EG I*, 8 ECU/l	43.41 (0.39)
		1.42
25	EG I*, 12 ECU/l	44.29 (0.35)
		2.30
30	EG I*, 20 ECU/l	44.34 (0.47)
		2.35

**EXAMPLE 2****Carbon dispersing effect of EG I\***

5 The particulate soil removing effect of endoglucanases is expected partly to be ascribed to the cleavage of glycosidic bonds in the cellulose matrix, but to some extent the enzyme may also provide a more non-specific cleaning effect, for instance, by improving the dispersability of the particulate  
10 soil.

In this test it is shown that EG I\* (truncated EG I, 402 amino acids, see Figure 3) differs from EG I (415 amino acids, WO 91/17244 Fig. 14A-E) with respect to its ability to disperse active carbon.

15

Different amounts of purified EG I and EG I\* were added into 10ml of a 1g/l suspension of active carbon (Norit) in 10mM Phosphate buffer, pH 10.

The mixtures were incubated for 30 minutes at 55°C and 150  
20 rpm.

After incubation the samples were allowed to cool to ambient temperature and non-dispersed carbon was allowed to settle (no centrifugation) for 15 minutes. The amount of carbon that was dispersed was evaluated by measuring the OD<sub>582nm</sub> of  
25 the supernatant (at 582nm was found a peak maximum most likely resulting from scattering). Due to the nature of the experiment (inhomogeneous solutions, time dependence etc), the absolute OD<sub>582nm</sub> levels may vary among the tests carried out, whereas the relative levels usually may be conserved.

30

The table below show the results of obtained in the test:

Enzyme	Conc. (mg/l)	$\Delta OD(582nm)$
EG I	5	0.09
	10	0.30
EG I*	5	0.40
	10	0.49

$$\Delta OD(582nm) = OD_{582nm}(\text{with enzyme}) - OD_{582nm}(\text{without enzyme})$$

$$5 \text{ } OD_{582nm}(\text{without enzyme}) = 0.716$$

The results show that EG I\* differs significantly from EG I in terms of its carbon dispersion ability - at 5mg/l level the  $\Delta OD(582nm)$  obtained with EG I\* is four times as high as that obtained with EG I. Also it should be noted that the effect of EG I\* is actually very large - the apparent level of detergency is increased by about 70% in the presence of 10mg/l EG I\*.

15

**EXAMPLE 2****Tensile strength loss induced by cellulase**

20 Cellulases used for soil removal in detergents often gives rise to an increased fabric wear. This can be observed through a reduced tensile strength of the fabric.

In the present example three cellulases are compared: Cellu-  
25 zyme<sup>®</sup> (a known commercial cellulase preparation), EG I\* from *H. insolens* (Figure 3) and EG I-Fus from *Fusarium* (Figure 4) (both cellulases of the present invention).

Celluzyne<sup>®</sup> is a multicomplex cellulase product from *Humicola*  
30 *insolens* used in detergents for soil removal and color clarification.

**Experimental:**

Buffer: 0.05 M Tris-HCl, pH 7.0, 1 mM CaCl<sub>2</sub>  
Textile/cup: 4 pcs. á 5 x 25 cm; woven fabric (app. 18 g)  
Dosage: 10000 ECU/l of  
5 Celluzyme®,  
*Humicola insolens* EGI PPC 4192,  
*Fusarium* EGI-161294/MChr.  
Volume: 100 ml  
Time: 7 days dark storage, 25 °C.  
10 Rinse: 10 min in deionized water.  
Evaluation: Tensile strength is measured as wet pull on Instron.

The following results were obtained:

15

	% tensile strength loss
Blind (no cellulase)	0% ± 8% **)
Celluzyme	41%
<i>H. insolens</i> EG I*	8%
20 <i>Fusarium</i> EGI-Fus	-4%

\*\* ) 0% pr. definition; 8% is relative standard deviation.

From the results it can be concluded that EG I\* from *H. in-*  
25 *solens* and EGI-Fus from *Fusarium oxysporum* are effective for soil removal but their use do not result in significant tensile strength loss in textile fabric.



## CLAIMS

1. A cellulase selected from the group consisting of Family 7 cellulases and variants of these cellulases, wherein said cellulase comprises a core and optionally further comprises a C-terminal link consisting of 10 amino acids at the most, provided that the cellulase is different from the endoglucanase having the amino acid sequence listed in Figure 1.
2. A cellulase according to claim 1, wherein the amino acid residue in the position corresponding to position 55 of the structural position numbering in Figure 5 is tryptophan, tyrosine or phenylalanine, preferably tryptophane.
3. A cellulase according to claim 1 or 2 which is obtainable by or derived from a strain of *Humicola*, *Trichoderma*, *Myceliophthora*, *Penicillium*, *Irpex*, *Aspergillus*, *Scytalidium* or *Fusarium*.
4. A cellulase according to claim 3, which is derivable from a strain of *Humicola insolens*, *Fusarium oxysporum*, *Myceliophthora thermophila* or *Trichoderma reesei*.
5. A cellulase according to claim 4, which is derivable from a strain of *Humicola insolens* and comprises the amino acid residues 1-397 listed in Figure 3 and optionally a C-terminal link consisting of 10 amino acids at the most, or a variant of said cellulase having an amino acid sequence being at least 60% homologous with said sequence, provided that the cellulase is different from the endoglucanase having the amino acid sequence listed in Figure 1.

6. A cellulase according to claim 4, which is derivable from a strain of *Fusarium oxysporum* and comprises the amino acid residues 1-401 listed in Figure 4 and optionally a C-terminal link consisting of 10 amino acids at the most, or a variant of said cellulase having an amino acid sequence being at least 60% homologous with said sequence.

7. A cellulase according to claim 4, which is derivable from a strain of *Trichoderma reesei* and comprises the amino acid residues 1-369 listed in Figure 2 (the amino acid sequence denoted Egltrire) and optionally a C-terminal link consisting of 10 amino acids at the most, or a variant of said cellulase having an amino acid sequence being at least 60% homologous with said sequence.

15

8. A cellulase according to claim 4, which is derivable from a strain of *Myceliophthora thermophila* and comprises the amino acid residues 1-456, preferably the residues 1-420, more preferably the residues 21-420, listed in Figure 6 and optionally a C-terminal link consisting of 10 amino acids at the most, or a variant of said cellulase having an amino acid sequence being at least 60% homologous with said sequence, provided that the cellulase is different from the endoglucanase having the amino acid sequence listed in Figure 1.

9. A cellulase having high activity on cellotriose in the presence of a detergent matrix, high dispersing action on carbon black, and high alkaline activity on acid swollen cellulose at pH 8.

10. A cellulase according to claim 9, wherein the activity on cellotriose in the presence of a detergent matrix corresponds to an apparent  $k_{cat}$  of at least 1 per sec at pH 8.

35

11. A cellulase according to claim 9, wherein the dispersing action on carbon black at pH 10 corresponds to a delta value of at least 0.20 measured at 582 nm for 5 mg/l of cellulase.
- 5 12. A cellulase according to claim 9, wherein the alkaline activity on acid swollen cellulose at pH 8 corresponds to an apparent  $k_{cat}$  of at least 10 per sec.
13. A cellulase according to any of the claims 5-8 having  
10 one or more insertions of between 1 and 25, preferably between 1 and 20, amino acid residues.
14. A cellulase according to claim 13, wherein the insertion is relative to EG1-Tat position 230-240 in EG1-H and EG1-F  
15 and part of which is located within 10Å of the active site residues E197, D199 and E202.
15. A cellulase according to any of the claims 1-14, wherein the depth of the substrate binding cleft is at least 12Å,  
20 preferably 15Å, when measuring between C $\alpha$  atoms of the active site residues located at the bottom of the cleft and C $\alpha$  atoms of residues located at the rim of the substrate binding cleft immediately above the active site residues.
- 25 16. A cellulase according to claim 5 or 6, wherein one or more of the following amino acid residues are substituted:  
N89Q  
N89Q+N247Q  
H123N  
30 T385N  
Q399N  
E202A  
S37W+P39W.
- 35 17. A cellulase according to claim 5, 6 or 17, wherein one or more of the following amino acid residues are substituted:

M142E  
K217A  
K217A+K218T  
R245G  
5 I310D  
E150Q  
E334K  
M198L.

10 18. A cellulase according to any of the claims 5, 6, 16 or 17, wherein one or more of the following amino acid residues within 5Å of bound cellobiose are substituted:

R106x, Y145x, S345x, D34x, W51x, S104x, A143x, Y171x, D173x,  
15 Q175x, Y177x, W347x, where x is chosen to modify H-bonding potential and/or hydrophobic interaction with the substrate.

19. A detergent composition comprising a cellulase according to any of claims 1-18 and a surfactant.

20

20. A detergent composition according to claim 19, wherein the cellulase is an endoglucanase derived from a strain of *Humicola insolens* and having the amino acid sequence listed in Figure 3 and an apparent molecular weight of about 50 kD  
25 measured in SDS-PAGE.

21. A detergent composition according to claim 19 or 20, which further comprises one or more other enzymes, in particular amylases, lipases, proteases, cellulase components,  
30 peroxidases, and/or oxidases.

22. A washing process wherein soiled fabric is treated with a cellulase according to any of the claims 1-18.

35 23. Use of a cellulase according to any of the claims 1-18 in detergent compositions or in fabric softener compositions in an amount which is effective for soil removal.

24. Use of a cellulase according to any of the claims 1-18  
for colour clarification of textile fabrics (removal of  
fluffs and pills), for preventing backstaining in washing of  
fabric, for soil removal of textiles, for bio-polishing of  
5 textiles, for deinking of used paper, for drainage improve-  
ment of paper pulp, for defibration of paper pulp or for de-  
barking of paper pulp.

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## Protein: Humicola insolens

## Amino acid sequence

```

-----
KPKETKEVH POLTFRCTK RGCCKPATNF IVLSLSHPH HRAEGLPGG CGDMGNPPPK DVCPDVESCA      70
KNCIMEGIPD YSQYGVTTNG TSLRLQHILP DGRVPSRPVY LLDKTKRREY MLHLTGFEFT FDVDATKLLPC      140
GMNSALYLSE MHPTGAKSKY NSGGAYVGTG YCDAQCFVTP FINGLGNIEG KGSCCNEMDI WEVNSRASHV      210
VPHTCNKKGL YLCEGECAF EGVCDKNGCG WNNYRVNVD YYGRGEFKV NTLKPFVTVT QFLANRRGKL      280
EKIHRFYVQD GKVIESFYTN KEGVPYTNMI DDEFCEATGS RKYRELGATQ GMGEALTRGM VLAHSIHWDO      350
GCNMEWLDHG EAGPCAKGEG APSNIQVQEP FPEVTTYTLR WGEIGSTYQE LQ                        402
-----

```

## Composition :

aa	#	%	aa	#	%
Xxx [X]:	0	0.00	Pro [P]:	23	5.72
Asp [D]:	18	4.48	Gly [G]:	47	11.69
Asn [N]:	22	5.47	Ala [A]:	18	4.48
Thr [T]:	28	6.97	Val [V]:	25	6.22
Ser [S]:	18	4.48	Cys [C]:	18	4.48
Glu [E]:	31	7.71	Met [M]:	11	2.74
Gln [Q]:	12	2.99	Ile [I]:	14	3.48
			Leu [L]:	26	6.47
			Tyr [Y]:	19	4.73
			Phe [F]:	15	3.73
			Lys [K]:	23	5.72
			His [H]:	10	2.49
			Trp [W]:	7	1.74
			Arg [R]:	17	4.23

Fig. 1

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Alignment		
Egltrire	QQPGETSTPEVHPKLTYYKCTKSGGCVAAQQTSSVVLdwnyrwm-----HNYSCTVNGGVN	54
Cendofus	-QTPDKAKEQHPKLETYRCTKASGCKKQTNIVADagihgir-----RSAGCGDWGQKPN	54
EGIHUM	-QKPGETKEVHPQLTTFRCTKRGCKKPATNFIVLdslshpihraeglgPGCGDWNPPPP	59
Egltrire	TTLCPEATCGKNCFIEGV---DYAASGVTTSGSSLTMNQYMPSSsggyssVSPRLYLDD	111
Cendofus	ATACPDEASCAKNCILSGmdsnAYKNAGITTSGNKLRQLQQLINNQL-----VSPRVYLLE	109
EGIHUM	KDVCPEVESCAKNCIMEGip--DYSQYGVTTNGTSLRLQHLILPDGgrv-----PSPRVYLDD	113
Egltrire	SD-GEYVMLKLNQELSFVDVLSALPCGENGSLYLSQMDENGGAN--QYNTAGANYGSGY	168
Cendofus	ENKKKYEMHLHGTCTEFSDVEMEKLP CGMGALYLSEMPQDGGKStsrNSKAGAYYGAGY	169
EGIHUM	KTKRRYEMHLHGTCTEFSDVEMEKLP CGMNSALYLSEMHPTGAKS---KYNPGGAYYGTGY	171
Egltrire	CDAQCPVQVTRNGTLNTSHQFCNEMDILEGNSRANALTPHSC-----TA	214
Cendofus	CDAQCYVTPFFINGVGNIKQGVCNCLDIWEANSRATHIAPHPCSKpglygctgdecgSS	229
EGIHUM	CDAQCFVTPFFINGLGNIEGKSGCCNEMDIWEVNSRASHVVPHTCNkkglylcegeecaFE	231
Egltrire	TACDSAdCGENPYGSGYKSYVGPGBdvdtsktftiitqfntdngspsgnlvsitryqqn	274
Cendofus	GICDKAGCGWNNRINVTDFYGRGkqykvdstrkftvtstsqfvanqgdlielhrhyiqdn	289
EGIHUM	GVCDKNGCGWNNYRVNVTDYVYGRGEefkvntlkpftvvtqflannrrgklekhrfyvqdg	291
Egltrire	gvdiipsaqpggdtisscpsasay-----GGLATMGKALSSGMVLVFSIWNDS	322
Cendofus	kviesavvni sgppkinfindkycaatganeymrlGGTKQMGDAMSRGMVLA MSVWVWSEG	349
EGIHUM	kviesfyt nkegvpytnmiddefceatgsrkymelGATQGMGEALTRGMVLA MSIWWDQDQ	351
Egltrire	QYMNWLD SGNAGPCSSSTEGNPSNLIANNPNTHVVFNSIRWGDIGSTTNSTAPPppasst	382
Cendofus	DFMAWLDQGVAGPCDATEGDPKNIVKVQPNPEVTFNSIRIGEIGSTSSVKAPAYPgphrI	409
EGIHUM	GNMEWLDHGEAGPCAKGEGAPSNIVQVEPFPPEVITYNLRWGEIGSTYQEVQKPKPkpghg	411
Egltrire	tfstttrrssttsspsctqthwggcggi gysgckctctsgtctcqysndyysqcl	435
Cendofus	-----	409
EGIHUM	prsd-----	415

Fig. 2

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Protein: *Mimicola insolens*

Amino acid sequence

-----	
QKPGETKEVH	60
PQLTTFRCYK	
RGCKPATNF	
IVLDSLHPH	
HRAEGLPGG	
CGDWGNPPPK	
DVCPDVECA	120
KNCIMEGIPD	
YSQYGVTTNG	
TSLRLQHILP	
DGRVPSPRVY	
LLDKTKRRYE	
MLHLTGFEFT	180
FDVDATKLPC	
GMNSALYLSE	
MHPTGAKSKY	
NPGGAYYGTG	
YCDAQCFVTP	
FINGLGNIEG	240
KGSCCNEMDI	
WEANSRASHV	
APHTCNKKGL	
YLCEGECECAF	
EGVCDKNGCG	
WNNYRVNVTD	300
YYGRGEEFKV	
NTLKPFTVVT	
QFLANRRGKL	
EKIHRFYVQD	
GKVIESFYTN	
KEGVPTNMI	360
DDEFCEATGS	
RKYMELGATQ	
GMGEALTRGM	
VLAMSIWWDQ	
GGNMEWLDHG	
EAGPCAKGEG	402
APSNIVQVEP	
FPEVYTNLR	
WGEIGSTYQE	
LQ	
-----	

Composition :

aa	#	%	aa	#	%	aa	#	%
Xxx [X]:	0	0.00	Pro [P]:	2	45.97	Leu [L]:	2	66.47
Asp [D]:	18	4.48	Gly [G]:	47	11.69	Tyr [Y]:	19	4.73
Asn [N]:	22	5.47	Ala [A]:	20	4.98	Phe [F]:	15	3.73
Thr [T]:	28	6.97	Val [V]:	23	5.72	Lys [K]:	23	5.72
Ser [S]:	17	4.23	Cys [C]:	18	4.48	His [H]:	10	2.49
Glu [E]:	31	7.71	Met [M]:	11	2.74	Trp [W]:	7	1.74
Gln [Q]:	12	2.99	Ile [I]:	14	3.48	Arg [R]:	17	4.23



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## Fusarium ENDO a EG i fam-C MATURE

One letter aminoacid codecode mature protein sequence

QTPDKAKEQH PKLETYRCTK ASGCKKQTNV IVADAGIHGI RQKNGAGCGD  
 WGQKPNATAC PDEASCAKNC ILSGMDSNAY KNAGITSGN KLRLQQQLINN  
 QLVSPRVYLL EENKKYEML HLTGTEFSFD VEMEKLPCGM NGALYLSEMP  
 QDGGKSTSRN SKAGAYYGAG YCDAQCYVTP FINGVGNKIG QGVCCNELDI  
 WEANSRATHI APHPCSKPGL YGCTGDECGS SGICDKAGCG WNNRINVTD  
 FYGRGQYKV DSTRKFTVTS QFVANKQGD L IELHRHVIQD NKVIESAVVN  
 ISGPPKINFI NDKYCAATGA NEYMLGGTK QMGDAMSRGM VLAMSVWVSE  
 GDFMAWLDQG VAGPCDATEG DPKNIVKVQP NPEVTFNIR IGEIGSTSSV  
 KAPAYPGPHR L

## Fusarium ENDO a EG i fam-C MATURE

Composition :

aa	#	%	aa	#	%	aa	#	%
Xxx [X] :	0	0.00	Pro [P] :	20	4.87	Leu [L] :	21	5.11
Asp [D] :	21	5.11	Gly [G] :	45	10.95	Tyr [Y] :	17	4.14
Asn [N] :	28	6.81	Ala [A] :	32	7.79	Phe [F] :	9	2.19
Thr [T] :	22	5.35	Val [V] :	21	5.11	Lys [K] :	31	7.54
Ser [S] :	26	6.33	Cys [C] :	18	4.38	His [H] :	9	2.19
Glu [E] :	20	4.87	Met [M] :	11	2.68	Trp [W] :	6	1.46
Gln [Q] :	18	4.38	Ile [I] :	22	5.35	Arg [R] :	14	3.41

Fig. 4

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SEQUENCE	10	20	30	40	50	60	Res
EG1, Fusariu	-QTPDKAKEQHPKLE	TYRCTKASGCKQTNY	IVADAGIHGIRQKN	---	GAGCGDWGQKPN		56
EG1, Humicol	-QKPGETKEVHPQLT	FRCTKRGGCKPATNF	IVLDLSLHP	IHRAEGLPGGCGDWGNPPP			59
EG1, T. rees	QQPGTSTPEVHPKL	TTYKCTKSGGCTVAQ	QTSVLDWNYRWMHNYNS	-----	CTVNGGV-N		54
CBH1, T. rees	XSACTLQSETHPPL	TWQKCSSGGTCTQ	QTQGTGSVVIDANWR	WTHATNS	---STNCYD-GNTWS		57
SEQUENCE	70	80	90	100	110	120	Res
EG1, Fusariu	ATACPDEASCAKNC	ILSGMDSNAYKN	-AGITTSGNKLR	LQQLI	---	N--NQLVSPRVYLL	110
EG1, Humicol	KDVCPDVESCAKNC	IMEGIP	--DYSQ-YGVTTNGT	SLRLQHILP	--D--	GRVPSPRVYLL	112
EG1, T. rees	TTLCPEATCGKNCF	IEGV	---DYAA-SGVTTSGSL	TWQYMPSSSGYSSVSPRLYLL			110
CBH1, T. rees	STLCPDNETCAKNC	CLDGA	---AYASTYGVTTSG	NSLSIGFVT	---QSAQKNVGARLYLM		111
SEQUENCE	130	140	150	160	170	180	Res
EG1, Fusariu	EENKKKYEMHLTG	TEFSFVEMEKLP	CGMNGALYLSE	MPQDGGKSTSRNS	KAGAYYGAG		170
EG1, Humicol	DKTKRRYEMHLTG	FEFTFDVDTKL	PCGMNSALYLSE	MHPTGAKS	--KYNPGGAYYGTG		170
EG1, T. rees	DSD-GEYVMLKNG	QELSFVDLSAL	PCGENGSLYLSQ	MDENGGAN	--QYNTAGANYGSG		167
CBH1, T. rees	ASD-TTYQEFTLL	GNEFSFVDVVSQ	LPCLNGALYFV	MDADGGVSKYPT	NTAGAKYGTG		170
SEQUENCE	190	200	210	220	230	240	Res
EG1, Fusariu	YCDAQCYV-TPFI	NGVGNK	-----	QQGVCCNELDI	WEANSRATHIAPHPC		215
EG1, Humicol	YCDAQCFV-TPFI	NGLGNIE	-----	GKGSCCNEMDI	WEANSRASHVAPHTC		215
EG1, T. rees	YCDAQCPV-QTWR	NGTLNTS	-----	HQGFCCNEMDI	LEGNSRANALTPHSC		212
CBH1, T. rees	YCDSCQPRDLKF	INGQANVEGWEP	SSNNANTGIG	HGSCCSEMDI	WEANSISEALTPHPC		230

Fig. 5 (Part I)

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SEQUENCE	250	260	270	280	290	300	Res
EG1, Fusariu	SKPGLYGCTGDECG-----	SSGICDKAGCGWNNHRINVTDFYGRGKQYKVDSTRKFTV					268
EG1, Humicol	NKKGLYLCEGECA-----	FEGVCDKNGCGWNNYRVNVTDYGRGEEFKVNTLKPFVV					268
EG1, T. rees	TAT---ACDSAGCG-----	FNPYSGYKSYGPGDTV--DTSKTFI					249
CBH1, T. rees	TTVGQICEGDCGCGTYSNRYGGTCDPDGCDWNPYRLGNTSFYGPSSFTLDTTKKLTIV						290
SEQUENCE	310	320	330	340	350	360	Res
EG1, Fusariu	TSQFVANKQ---GDLIELHRHYIQDNKVIESAVVNISGPPKINFINDKYCAATGA----	N					321
EG1, Humicol	VTQFLANRR---GKLEKIHRFYVDQGVKLVIESFYTNKEGVPTNMIDDEFCEATGS----	R					321
EG1, T. rees	ITQFNTDNGSPSGNLVSIITRKYQQNGVDIPSA-----	QPGGDTISSCPSASA-----					296
CBH1, T. rees	VTQFETS-----GAINRYVYVQNGVTFFQQPNAEL-GSYSGNELNDDYCTAEAEAFGGS						341
SEQUENCE	370	380	390	400	410	420	Res
EG1, Fusariu	EYMRLLGCTKQMGDAMSRGMVLAMSVWVWSEGDFMAWLDQG-----	VAGPCDATE					369
EG1, Humicol	KYMEIGATQGMGEALTRGMVLAMSIWWDQGGNMEWLDHG-----	EAGPCAKGE					369
EG1, T. rees	----YGGLATWKGKALSSGMVLVFSIWNDNSQYMNWLDG-----	NAGPCSSTE					340
CBH1, T. rees	SFSDKGGTLTQFKKATSGGMVLVMSLWDDYYANMLWLDSTYPTNETSSTPGAVRGSCSTSS						401
SEQUENCE	430	440	450	460	470	480	Res
EG1, Fusariu	GDPKNIKVQNPPEVTFNSNIRIGEIGSTSSVKAPAYPGPHRL						411
EG1, Humicol	GAPSNIVQVEPFPPEVITYNLRWGEIGSTYQEVQKPKPKPGHGRSD						415
EG1, T. rees	GNPSNILANNPNTHVVFNSNIRWGDIGSTTNTSTAPPPPPASSTTFST						386
CBH1, T. rees	GVPAQVESQSPNAKVTFNSNIKFGPIGSTGNPSG						434

Fig. 5 (Part II)

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Amino acid sequence of endoglucanase from *Myceliophthora thermophila* :

1  
MGRGAAFLGL ASLLVGAAGA QTPGEGEEVH PQITTYRCK 40  
ADGCEEKTNV IVLDALSHPV HQVDNPYNCG DWGQKPNETA 80  
CPDLESCARN CIMDPVSDYG RHGVSTDGTS LRLKQLVGGN 120  
VVSPRVYLLD ETKERYEMLK LTGNEFTFDV DATKLPCGMN 160  
SALYLSEMDA TGARSELNPG GATFGTGYCD AQCYYVTPFIN 200  
GLGNIEGKGA CCNEMDIWEA NARAQHIAPH PCSKAGPYLC 240  
EGAECEFDGV CDKNGCAWNP YRVNVTDYG EGAEFRVDTT 280  
RPFSVVTQFR AGGDAGGGKL ESIYRLFVQD GRVIESYVVD 320  
KPGLPPTDRM TDEFCAATGA ARFTELGAME AMGDALTRGM 360  
VLALSIWWSE GDNMNWLDSE EAGPCDPDEG NPSNIIRVQP 400  
DPEVVFSNLR WGEIGSTYES AVDGPVGKGR GKGKGKAPAG 440  
DGNGKEKSNG KRFRRF 456

The protein sequence starts at position 21.

The last 36 amino acid residues form a tail which will be removed by proteases.

Fig. 6

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00108

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC6: C12N 9/42 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0495257 A1 (THE PROCTER & GAMBLE COMPANY), 22 July 1992 (22.07.92), see page 5, line 55-page 11, line 14 and in particular page 9, lines 37-38  --	1-5,13-15, 19-24
X	EP 0495258 A1 (THE PROCTER & GAMBLE COMPANY), 22 July 1992 (22.07.92), page 11, line 40 - line 43  --	1-5,13-15, 19-24
X	WO 9117243 A1 (NOVO NORDISK A/S), 14 November 1991 (14.11.91), page 6, line 31 - page 7  --	1-5,13-15, 19-24
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
19 June 1995		25 -07- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer  Carl Olof Gustafsson Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00108

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9305226 A1 (UNIVERSITY OF BRITISH COLUMBIA), 18 March 1993 (18.03.93), see pages 8-12 and claim 1 and 9, see in particular page 12, lines 20-31 --	1-5,13-15, 19-24
A	EP 0540784 A1 (THE PROCTER & GAMBLE COMPANY), 12 May 1993 (12.05.93), see the whole document --	1-5,13-15, 19-24
A	TIBTECH, Volume 7, Sept 1989, Edgar Ong et al, "The cellulose-binding domains of cellulases: tools for biotechnology", page 239, see the whole document --	1-5,13-15, 19-24
P,X	WO 9407998 A1 (NOVO NORDISK A/S), 14 April 1994 (14.04.94), page 3, line 26 - page 4, line 17; page 9, line 31 - page 10, line 9, claims --	1-5,13-24
X	WO 9117244 A1 (NOVO NORDISK A/S), 14 November 1991 (14.11.91), see example 3, figure 13 and claim 11 --	1-4,6,13-24
A	see example 4, figure 14 and claim --	1-4,8,13-15, 19-24
X	Biotechnology, Volume 5, January 1987, Janelle N. Van Arsdell et al, "Cloning, Characterization, and Expression in Saccharomyces Cerevisiae of Endoglucanase i from Trichoderma Reesei", page 60, see fig 1 and "Discussion" --	1-4,7,13-15, 19-24
X	EP 0137280 A1 (CETUS CORPORATION), 17 April 1985 (17.04.85), page 66 - page 67 --	1-4,7,13-15, 19-24

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK 95/00108

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 154, Medline, Dialog accession No. 06132822, Medline accession No. 87106822, Penttila M. et al: "Homology between cellulase genes of Trichoderma reesei: complete nucleotide sequence of the endoglucanase I gene", Gene (NETHERLANDS) 1986, 45 (3) p 253-63  --	1-4,7,13-15, 19-24
X	WO 8504672 A (VALTION TEKNILLINEN TUTKIMUSKESKUS), 24 October 1985 (24.10.85), page 14 - page 17, figure 6, claim 5  --	1-4,7,13-15, 19-24
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International application No.  
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00108

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

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1. A cellulase and applications thereof according to claim 1-5 and 13-24 from *Humicola insolens* comprising the amino acid sequence listed in fig. 1.
2. A cellulase and applications thereof according to claims 1-4,6 and 13-24 from *Fusarium oxysporum* comprising the amino acid sequence listed in fig 4.
3. A cellulase and applications thereof according to claims 1-4,7,13-15 and 19-24 from *Trichoderma reesei* comprising the amino acid sequence listed in fig 2.
4. A cellulase and applications thereof according to claims 1-4,8,13-15 and 19-24 from *Myceliophthora thermophila* comprising the amino acid listed in fig 6.
5. A cellulase and applications thereof having high activity on cellotriose in the presence of a detergent matrix according to claims 9-12 and 19-24.

